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# Mutational analysis of genes implicated in LPS and capsular polysaccharide biosynthesis in the opportunistic pathogen *Bacteroides fragilis*

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The obligate anaerobe *Bacteroides fragilis* is a normal resident of the human gastrointestinal tract. The clinically derived *B. fragilis* strain NCTC 9343 produces an extensive array of extracellular polysaccharides (EPS), including antigenically distinct large, small and micro- capsules. The genome of NCTC 9343 encodes multiple gene clusters potentially involved in the biosynthesis of EPS, eight of which are implicated in production of the antigenically variable micro-capsule. We have developed a rapid and robust method for generating marked and markerless deletions, together with efficient electroporation using unmodified plasmid DNA to enable complementation of mutations. We show that deletion of a putative wzz homologue prevents production of high-molecular-mass polysaccharides (HMMPS), which form the micro-capsule. This observation suggests that micro-capsule HMMPS constitute the distal component of LPS in *B. fragilis*. The long chain length of this polysaccharide is strikingly different from classical enteric O-antigen, which consists of short-chain polysaccharides. We also demonstrate that deletion of a putative wbaP homologue prevents expression of the phase-variable large capsule and that expression can be restored by complementation. This suggests that synthesis of the large capsule is mechanistically equivalent to production of *Escherichia coli* group 1 and 4 capsules.

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## INTRODUCTION

Encapsulating polysaccharides surrounding bacteria have diverse roles in protecting the cell against environmental insults, for example prevention of desiccation, resistance to bacteriophage infection, and avoidance of host immune systems (Sutherland, 1977). Capsule production in *Escherichia coli* is the paradigm for understanding the genetics and biochemistry of extracellular polysaccharide (EPS) production. In both *E. coli* and *Salmonella enterica*, polysaccharide biosynthetic genes are clustered together for the production of either the O-antigen of lipopolysaccharide (LPS) or capsular polysaccharides (groups 1, 2, 3 and 4; reviewed by Whitfield, 2006). Polysaccharides required for the production of LPS and capsules of groups 1 and 4 are transported across the inner membrane and polymerized by specific Wzx (flippase) and Wzy (polymerase) proteins, respectively. However, the fate of the polysaccharide is dictated by subsequent protein partners, which either ligate the polysaccharide to lipid A-core (WaaL for LPS and K<sub>LPS</sub>

production) or transport it directly beyond the outer membrane (Wza for capsular polysaccharides). In contrast, group 2 and 3 capsular polysaccharides are synthesized by processive glycosyltransferases and exported via an ABC transporter system. Despite the large number of serotypes found in *E. coli*, there is generally little intra-strain variation in capsular types; for example, a strain will only produce a single group 1 capsule, and not multiple variants, because there is only one polysaccharide biosynthetic locus present in the chromosome. The exception is colanic acid, which may be co-expressed with group 2 capsules (Whitfield, 2006). Where intra-strain antigenic variation does occur, two different polysaccharides may be generated by modification of the polysaccharide sugar moieties, for example by acetylation (Vimr & Steenbergen, 2006).

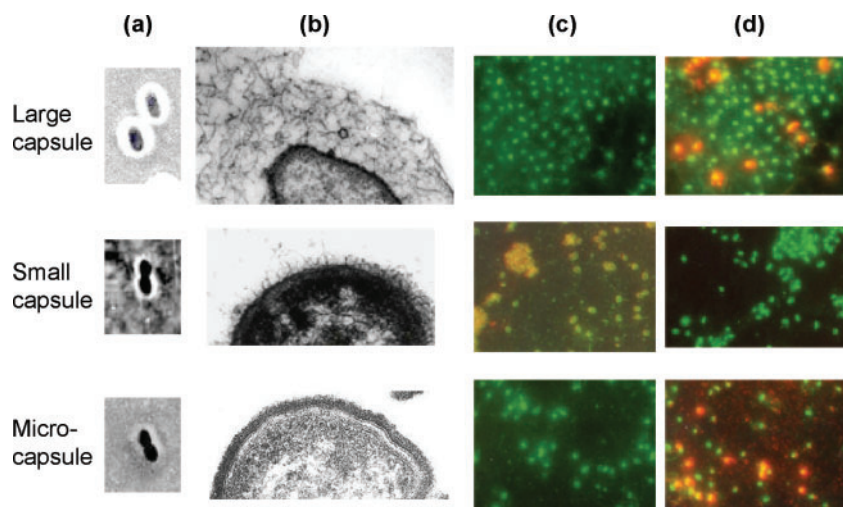
In contrast, the genome of *Bacteroides fragilis* NCTC 9343 contains ten annotated regions (PS A–J) implicated, by homology, in EPS production (Cerdeño-Tárraga *et al.*, 2005). PS loci A–I contain genes predicted to encode Wzx and Wzy proteins, suggesting that these polysaccharides will either be linked to lipid A-core prior to export or be secreted directly beyond the outer membrane. However, there are no annotated homologues for WaaL and only one potential Wza homologue can be found in the genome.

**Abbreviations:** EPS, extracellular polysaccharide(s); HMMPS, high-molecular-mass polysaccharide; IFM, immunofluorescence microscopy; LC, large capsule; MC, micro-capsule; PCP, polysaccharide copolymerase; R/M, restriction/modification; SC, small capsule.

*B. fragilis* is the Gram-negative obligately anaerobic member of the normal human intestinal microbiota most frequently isolated from opportunistic infections (Patrick, 2002; Patrick & Duerden, 2006). Infections include peritonitis, serious gynaecological sepsis, soft tissue abscess and bacteraemia. The latter has an estimated mortality of 19% (Redondo *et al.*, 1995). Potential virulence determinants include expression of within-strain phase and antigenically variable polysaccharides that form a marginal electron-dense layer, or micro-capsule (MC), of approximately 35 nm in size, outwith the outer membrane and not visible by light microscopy (Fig. 1; Patrick *et al.*, 1986; Lutton *et al.*, 1991). Of the eight PS loci (A–H) that are related to variable MC expression, seven are switched ON and OFF by the site-specific inversion of promoter sequences, where recombination can be mediated by two members of the serine family of invertases (Patrick *et al.*, 2003; Coyne *et al.*, 2003; Liu *et al.*, 2008). In addition to MCs, antigenically distinct and within-strain variable large capsules (LCs) and small capsules (SCs) are visible by light microscopy when cultures are grown in a glucose-defined medium (Fig. 1; Patrick *et al.*, 1986), for which the gene loci remain to be assigned. Populations of *B. fragilis* enriched for the different capsules can be obtained by using Percoll density-gradient centrifugation. The antigenically variable MCs are co-expressed with the LC; an electron-dense layer adjacent to the outer membrane is visible beneath the LC by electron microscopy, and reactivity with MC-specific mAbs is demonstrable by immunofluorescence microscopy (Fig. 1; Lutton *et al.*, 1991; Patrick, 1993). With continuous daily subculture these populations gradually revert to mixed capsular types. The LC confers resistance to phagocytic uptake and killing by human polymorphonuclear leukocytes *in vitro* (Reid & Patrick 1984), whereas MC-enriched populations are phagocytosed and killed. The MC is also important for colonization of the mammalian gastrointestinal tract. Recent evidence suggests that a single polysaccharide is sufficient to allow effective competition of a mutant strain with wild-type *B.*

*fragilis* when co-inoculated into a gnotobiotic mouse (Coyne *et al.*, 2008). This finding, however, is contradicted by other evidence that suggests strains expressing a single polysaccharide are not competitive in the gastrointestinal tract (Liu *et al.*, 2008).

Genetic manipulation of many bacteria, including clinically relevant *B. fragilis* strains, has been problematic largely because of the restriction/modification (R/M) systems that recognize and cleave foreign DNA, which has usually been prepared from an *E. coli* host (Salyers *et al.*, 2000). *B. fragilis* NCTC 9343 contains a shufflon that can generate eight different HsdS proteins that provide DNA target specificity for a type I R/M system, plus two further type I and two type III R/M systems (Cerdeño-Tárraga *et al.*, 2005). Production of mutations in some strains of *B. fragilis*, often the 638R background, has been reported. One method relies on integration of the altered sequence into the chromosome followed by screening for spontaneous resolution of the diploid to yield the desired product (Coyne *et al.*, 2003, 2008). This approach can be time consuming and does not always produce the required mutant if the diploid is stable. An alternative approach uses resistance to trimethoprim, conferred by a chromosomal *thyA* mutation in *B. fragilis*, to select for resolution of diploids where the suicide plasmid carries a functional copy of *thyA* (Baughn & Malamy, 2002). While this method enriches resolved diploids, it has the drawback that the strain to be engineered must first be made trimethoprim resistant and so all resulting strains contain a *thyA* mutation, which may have undesirable consequences for further studies. For example, a *thyA* mutation in *E. coli* will induce the SOS response if the cells are not provided with exogenous thymine (Begg & Donachie, 1978). Here we describe and validate a new and robust method for generating deletion mutants, and the complementation thereof, in *B. fragilis* NCTC 9343. This technique enables the rapid and reliable production of mutants that are either marked with an antibiotic resistance determinant or are



**Fig. 1.** LC, SC and MC populations of *B. fragilis* prepared by discontinuous Percoll density-gradient centrifugation. (a) Light micrographs of negative capsule stain. (b) Transmission electron micrographs of ultrathin sections. (c, d) Immunofluorescence micrographs of populations labelled with rabbit anti-*B. fragilis* polyclonal antiserum, secondary labelled with anti-rabbit FITC-conjugated antibody and either mAb QUBf4 (c) or QUBf5 (d), with secondary labelling using anti-mouse tetramethyl rhodamine isothiocyanate conjugated antibody as detailed by Lutton *et al.* (1991).

markerless. The ability to complement non-polar deletions greatly facilitates genetic studies of this important human commensal and opportunistic pathogen. We also present direct evidence that two additional loci, PSJ and a region upstream of PSI, are involved in MC and LC biosynthesis respectively. These findings have implications for understanding how polysaccharides are synthesized in *B. fragilis*.

## METHODS

**Strains, plasmids and culture methods.** *B. fragilis* NCTC 9343 was obtained from the National Collection of Type Cultures, Colindale Avenue, London, UK. The GenBank accession number for the genome sequence of *B. fragilis* NCTC 9343 is NC\_003228. *E. coli* DH5 $\alpha$  (Invitrogen) was used for constructing plasmids using standard molecular biology protocols. Conjugative vectors for introducing DNA into *B. fragilis* were based on pEP185.2 and were mobilized from the *E. coli* S17-1 $\lambda$ pir strain (Simon *et al.*, 1983). Plasmid pLyl01 (Li *et al.*, 1995) was used to generate the I-SceI-expressing plasmid pGB920. Plasmid pVA2198 (Fletcher *et al.*, 1995) was used to produce plasmids containing the locked ON and OFF promoter BF2782 constructs. Media for cultivation of *B. fragilis* included supplemented brain heart infusion (BHI-S) and defined medium (DM; van Tassell & Wilkins, 1978). Cultures were grown in a MACS MG-1000 or Min-MACS anaerobic workstation (Don Whitley), at 37 °C in an atmosphere of 80 % nitrogen, 10 % carbon dioxide and 10 % hydrogen. *E. coli* was cultured on LB at 37 °C.

**Generation of deletion mutants.** Approximately 500 bp of DNA flanking the sequence to be deleted was amplified by PCR using *Pfu* polymerase (NEB). Flanking regions were fused to an *ermF* cassette by cross-over PCR using *Pfu* polymerase. PCR products were ligated into the multiple cloning site of pGB909 (a pEP185.2 derivative containing an I-SceI recognition sequence inserted at the *SacI* site). Ligated products were recovered in an *E. coli* S17-1 $\lambda$ pir strain. Constructs were mobilized from *E. coli* S17-1 $\lambda$ pir into *B. fragilis* NCTC 9343 using a filter-mating method (Valentine *et al.*, 1988), followed by selection on BHI-S plates containing 10  $\mu$ g erythromycin ml<sup>-1</sup>. Strains with integrated plasmids were confirmed by PCR and then electroporated with a derivative of pLyl01 containing the I-SceI coding region under the control of the *B. fragilis* *fucR* promoter, with selection for resistance to tetracycline. Transformants were streaked onto DM containing fucose and tetracycline. Colonies were then screened for resistance to erythromycin and deletion of the appropriate sequence confirmed by PCR. Markerless deletions were constructed by ligating cross-over PCR products from just the flanking regions into pGB910 (a derivative of pGB909 with an *ermF* cassette in the *KpnI* site). Conjugation and selection were as described above, except the final deletions were screened for loss of resistance to erythromycin.

**Percoll density centrifugation.** Discontinuous Percoll gradients were prepared as previously described (Patrick & Reid, 1983). A 100 % stock Percoll (Pharmacia Biosystems) suspension was prepared by diluting sterile Percoll 9:1 with sterile 1.5 M sodium chloride, and adjusting the pH to 7.0 using 1 M hydrochloric acid. Percoll suspensions of 20 % and 40 % (v/v) were prepared by diluting the 100 % stock with sterile 0.15 M sodium chloride. Cells grown in DM were layered on top of the gradients followed by centrifugation at 2375 g for 40 min at 4 °C. Subculture of bacteria separating at and above the 0–20 % interface results in enrichment of the large capsule population. Bacteria separating at the 20–40 % interface results in enrichment of the small capsule population, whereas bacteria that pass further down the gradient are enriched for the MC.

**Immunoblotting of EPS.** Immunoblots were prepared as previously described (Patrick *et al.*, 2003). Aliquots of DM cultures, in late exponential phase, were resuspended in 1/4 diluted sample buffer [40 % (w/v) glycerol, 0.25 M Tris/HCl pH 6.8, 20 % (w/v)  $\beta$ -mercaptoethanol, 16 % (w/v) SDS and 0.04 % (w/v) bromophenol blue] and heated at 100 °C for 10 min. Then 25  $\mu$ g proteinase K (Sigma) was added and incubated at 60 °C for 1 h. Digests were electrophoresed through Novex 10 % Tris/glycine polyacrylamide gels (Invitrogen) in Novex 1  $\times$  Tris/glycine SDS running buffer (25 mM Tris base, 192 mM glycine and 0.1 % SDS pH 8.3). Polysaccharides were transferred to nitrocellulose membranes by electroblotting in transfer buffer [12 mM Tris base, 96 mM glycine, 20 % (v/v) methanol, pH 8.3]. Membranes were blocked for 1 h at 37 °C in blocking buffer [1  $\times$  TBS, 5 % (w/v) dried semi-skimmed milk, 0.05 % (v/v) Tween-20], followed by washing in TBS-0.05 % Tween-20 (TBST) at 37 °C. Membranes were incubated with mAbs QUBf4 (Reid *et al.*, 1987), 5, 6, 7, 8 (Lutton *et al.*, 1991), 11 (Patrick *et al.*, 1995), 18, 19 (Patrick, 1997), 25 (unpublished) and CE3 (Pantosti *et al.*, 1995), for 1 h at 37 °C, followed by washing in TBST at 37 °C. The membrane strips were incubated for 1 h at 37 °C with either goat anti-mouse IgG (H + L) alkaline phosphatase conjugate (Bio-Rad) or goat anti-mouse IgM ( $\mu$  chain specific) alkaline phosphatase conjugate (Sigma). The membranes were washed in TBST and polysaccharides visualized by detecting alkaline phosphatase activity using Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium).

**Transformation of *B. fragilis* NCTC 9343.** Overnight cultures of *B. fragilis* were diluted in pre-reduced BHI-S broth and grown, anaerobically at 37 °C, to an OD<sub>600</sub> of 0.3. Cells were harvested by centrifugation at 1610 g for 20 min and washed five times in 20 ml pre-reduced ultrapure ice-cold water. Cells were finally resuspended in 1 ml pre-reduced ultrapure ice-cold water. One hundred microlitres of cells was used per electroporation in 0.2 cm electrode gene pulser cuvettes (Bio-Rad). If appropriate, diluted purified bacteriophage T7 Ocr (Overcomes classical restriction) protein, kindly gifted by Dr D. Dryden, School of Chemistry, University of Edinburgh, was added to the cells at the optimized concentration as detailed below. Then 500 ng plasmid DNA was added to the cells to give a final plasmid DNA concentration of 4.0  $\mu$ g ml<sup>-1</sup>. Electroporation was performed using a Gene Pulser II electroporator (Bio-Rad) at 2.5 kV for 5–10 ms. Cells were resuspended in 1 ml pre-reduced 37 °C BHI-S broth and incubated anaerobically for 2–3 h at 37 °C prior to plating on BHI-S plates with appropriate antibiotics.

**Capsule visualization.** *B. fragilis* possessing LC, SC and MC were identified using a negative capsule stain (Cruickshank, 1965). A 10  $\mu$ l drop of culture was pipetted onto a glass slide. Five microlitres of carbol fuchsin (Sigma) was added to the bacterial suspension, mixed, and incubated at room temperature for 30 s. Then 5  $\mu$ l eosin (Sigma) was applied and incubated at room temperature for 60 s. The stained cells were then smeared evenly across the slide using the edge of a microscope slide and air-dried at room temperature before visualization by microscopy. Cells and the background stained red, whereas capsules remain unstained. Transmission electron microscopy of ultrathin sections was carried out as previously described (Patrick *et al.*, 1986).

**Immunofluorescence microscopy (IFM).** IFM was performed as previously described (Patrick *et al.*, 2003). Cultures of *B. fragilis* grown in DM broth or BHI-S broth were diluted in sterile PBS to an OD<sub>600</sub> of 0.3. Then 10–30  $\mu$ l was pipetted onto the wells of a 12-well Teflon-coated slide (ICN Biomedicals) and dried at 37 °C, followed by fixing with 100 % methanol at –20 °C for 20 min. Slides were brought to room temperature; mAbs were applied to each well and then incubated at 37 °C for 1 h. Slides were rinsed in a stream of sterile PBS and washed in a PBS bath for 20 min. Slides were removed and secondary antibody (goat anti-mouse IgG/IgM-FITC conjugate).

Evans blue (0.1 %, Sigma), which stains proteins, was also added to each well as a counter-stain. Slides were then incubated for 1 h at 37 °C. The PBS washing step was repeated, and the slides were mounted in a glycerol-PBS antibleaching mounting fluid (Citifluor, Agar Scientific). Cells were examined using a Leitz Ortholux fluorescence microscope. Images were captured and analysed using an attached Nikon DMX 1200 digital camera and Lucia G/F software.

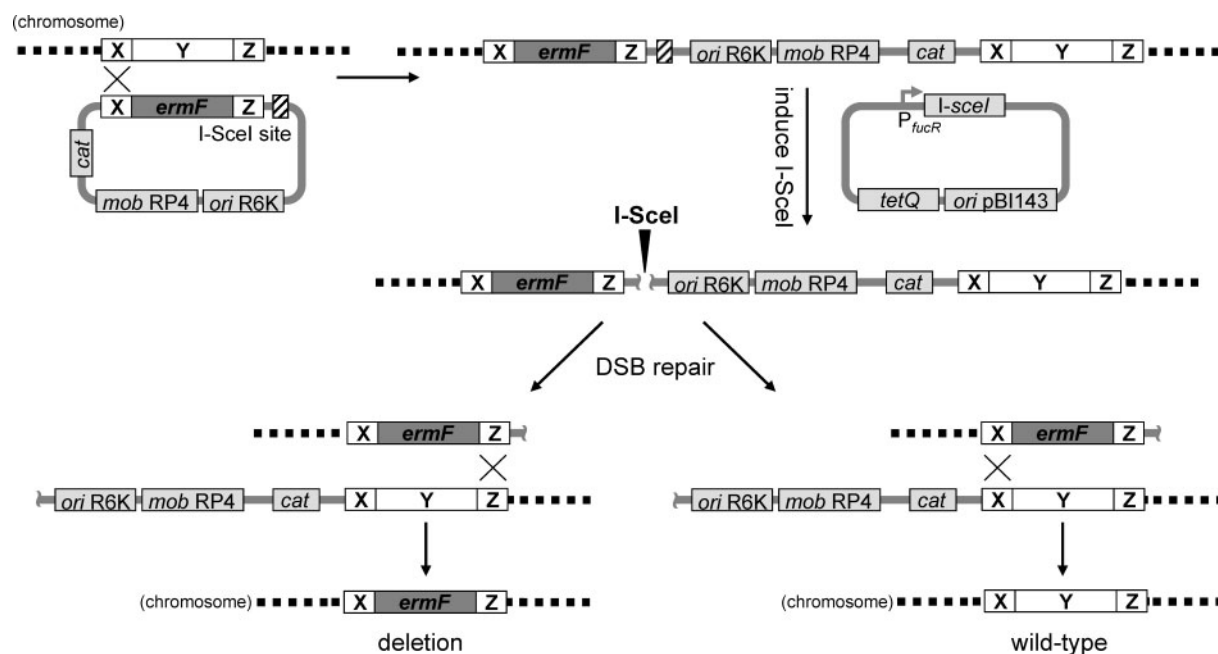
## RESULTS

### Using double-strand break repair to generate precise deletions in *B. fragilis*

To facilitate the manipulation of the sequenced strain of *B. fragilis*, NCTC 9343, we have adapted the method of Pósfai *et al.* (1999) to allow construction of deletions that are either marked by an antibiotic resistance determinant or are markerless. Marked deletion constructs are prepared by fusing 500 bp sequences from each side of the target gene, using PCR, to an intervening *ermF* cassette. The amplicon is ligated with an RP4-based conjugative vector (pEP185.2), into which we have inserted an ISce-I recognition sequence, prior to transformation of an appropriate *E. coli* host. The resulting strains are then conjugated with *B. fragilis* followed by growth in the presence of erythromycin to select for integration of the plasmid (Fig. 2). To resolve the diploid, the strains are

transformed with a plasmid that expresses the ISce-I meganuclease under the control of the *B. fragilis* *fucR* promoter, followed by growth on a defined medium that contains fucose. Resolution of the diploid by homologous recombination follows the ISce-I-mediated double-strand break and results in production of either the wild-type or the deletion genotype (Fig. 2). Colonies are then screened for resistance to erythromycin and the presence of the deletion confirmed by PCR. The advantage of this method is that it enhances resolution of the diploid and so most transformants are either mutant or wild-type; this removes the need to repeatedly subculture diploid strains and screen large numbers of colonies for resolution by random recombination events.

To construct markerless deletions, the sequences flanking the target gene are fused to each other and the amplicon ligated to a conjugative plasmid that contains an *ermF* cassette and an ISce-I recognition sequence. *E. coli* strains containing recombinant plasmids are conjugated with *B. fragilis* and integrants are selected by resistance to erythromycin, followed by resolution of the diploid by growth in the presence of fucose with subsequent screening for loss of erythromycin resistance. The resulting strains are then analysed by PCR to identify mutants containing the required deletion. The advantages of markerless deletions are that mutations should not be polar and they can be



**Fig. 2.** Generation of marked chromosomal deletions in *B. fragilis*. A suicide plasmid, containing an I-SceI recognition site (hatched box) and sequences homologous to chromosomal DNA (X and Z) flanking an *ermF* cassette and replacing the gene to be deleted (Y), is introduced into *B. fragilis* by conjugation. Plasmid integrants are selected for resistance to erythromycin. Diploids are transformed with a plasmid expressing I-SceI under the control of the fucose-inducible promoter  $P_{fucR}$ . Growth on defined medium in the presence of fucose leads to an I-SceI-mediated double-strand break (▼) and resolution of the diploid by generation of either the deletion or wild-type genotypes by homologous recombination. Chromosomal DNA is indicated as dashed black lines; plasmid DNA is indicated as solid grey lines.

complemented using plasmids bearing the same antibiotic resistance determinant, i.e. *ermF*; this is important since the natural resistance of *B. fragilis* to many antibiotics reduces the number of markers that can be used for selection.

Transformation has not been previously reported for *B. fragilis* NCTC 9343, probably as a result of the multiple R/M systems specified by the genome. We therefore determined if the inclusion of purified T7 anti-restriction protein Ocr acted as a transformation-enhancing co-factor by inhibiting the action of the extensive endogenous R/M systems of *B. fragilis*. The effect of various concentrations of Ocr protein on the number of transformants was compared to the transformation efficiency in the absence of Ocr. Standard electroporations using 500 ng pVA2198 plasmid DNA per transformation, optimized in the absence of Ocr, generated  $1.9 \times 10^4$  ( $\pm 1.9 \times 10^3$ ) transformants per  $\mu\text{g}$  unmodified DNA. The number of transformants obtained following electroporation increased steadily with increased Ocr protein concentration, reaching between  $5.0 \times 10^5$  ( $\pm 2.5 \times 10^4$ ) and  $5.2 \times 10^5$  ( $\pm 2.2 \times 10^4$ ) transformants  $\mu\text{g}^{-1}$ , corresponding to 16  $\mu\text{g}$  and 8  $\mu\text{g}$  Ocr protein per plasmid transformation. When Ocr concentration was increased from 16  $\mu\text{g}$  to 32  $\mu\text{g}$  protein per transformation, a tenfold decrease in transformant numbers was observed. An extensive selection of negative controls and transformant identification tests were performed, confirming that all erythromycin-resistant colonies identified were *B. fragilis* NCTC 9343 transformed with plasmid pVA2198. Therefore 16  $\mu\text{g}$  purified T7 Ocr anti-restriction protein ( $127 \mu\text{g ml}^{-1}$ ) was used per transformation. It has been shown that Ocr protein is capable of inhibiting type I R/M systems covering a diverse range of eubacteria and archaea, indicating that the anti-restriction property of the protein is not dependent on the target DNA sequence of the type I R/M enzymes (Krüger *et al.*, 1977, 1983; Walkinshaw *et al.*, 2002), as each type I system recognizes a unique target DNA sequence (Titheradge *et al.*, 2001). It is likely that the negative charge of this DNA mimic allows electroporation of the protein into the cell, where it is able to reduce the activity of the resident type I R/M systems (Hoffman *et al.*, 2002; Dryden, 2006). The all-encompassing type I anti-restriction activity of Ocr may therefore explain why the protein appears to be capable of improving transformation efficiency in *B. fragilis* NCTC 9343 by counteracting the DNA-degrading effects of all three complete type I R/M systems, including the phase-variable system.

### Validation of the deletion technique by disruption of polysaccharide gene cluster PSC

To validate our deletion strategy, we chose to replace the first gene in PSC, *upcY*, with an *ermF* cassette. Krinos *et al.* (2001) had previously shown that disruption of *upcY*, by insertion of a non-replicating plasmid, prevented expression of the associated polysaccharide. Expression

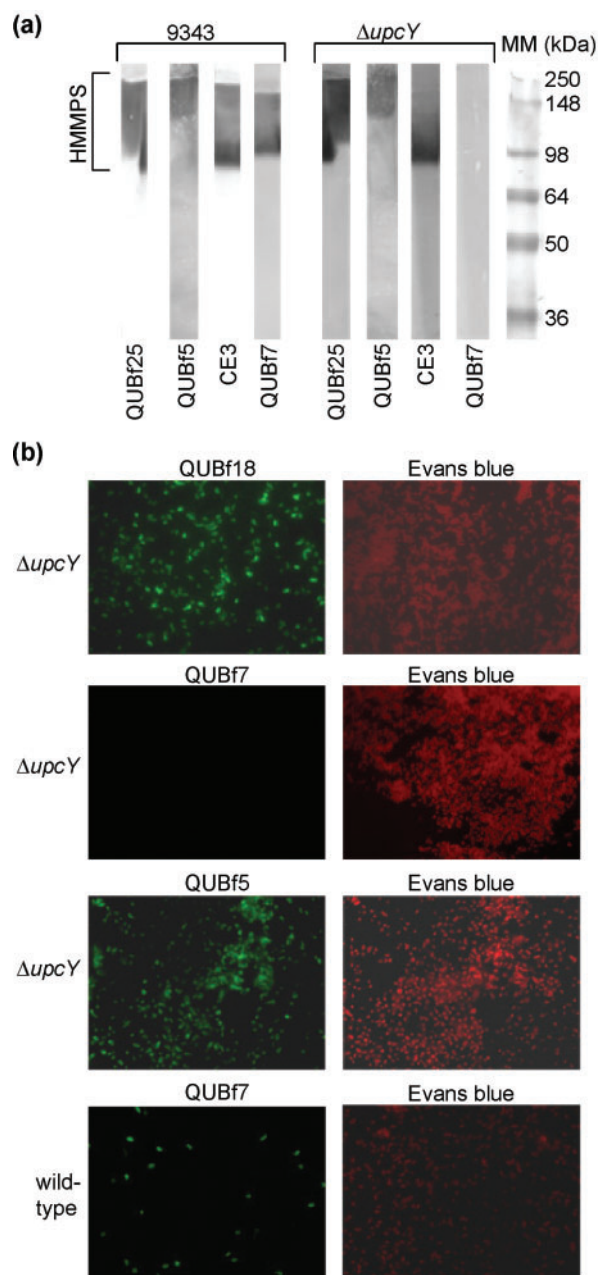
of PSC is not controlled by an invertible promoter and its regulatory mechanisms are currently unknown. However, the protein encoded by *upcY* is predicted to contain a KOW domain and has homology to NusG-like proteins; it is therefore likely to be involved in anti-termination of transcription within the leader sequence of the polysaccharide cluster.

We analysed the  $\Delta\text{upcY}::\text{ermF}$  strain by immunoblotting, IFM and negative staining of capsules using a collection of mAbs that specifically bind to distinct polysaccharides produced by *B. fragilis* NCTC 9343 (Patrick *et al.*, 2003; Fig. 3). Polysaccharides from the wild-type and mutant strains were prepared by proteinase K digestion of whole cells followed by electrophoresis through 12–20 % polyacrylamide gels. Immunoblots, using all mAbs, allowed detection of characteristic heteropolymorphic, high-molecular-mass polysaccharides (HMPS) produced by the parental strain, as described previously (Lutton *et al.*, 1991). Polysaccharides derived from the  $\Delta\text{upcY}::\text{ermF}$  strain were detectable on immunoblots using eight of the mAbs, but not QUBf7 (Fig. 3a). Binding of this mAb has previously been associated with polysaccharides produced by gene cluster PSC (Coyne *et al.*, 2000). IFM using the same mAbs confirmed that deletion of *upcY* led to an inability to detect cell-surface-associated polysaccharides that reacted with QUBf7; however, expression of other polysaccharides was unaffected (Fig. 3b). Both the LC and SC could be detected by negative staining of capsules (data not shown), indicating that only production of the PSC MC was affected by the mutation. It is possible that insertion of *ermF* has a polar effect on transcription, which subsequently leads to this phenotype. Together these data confirm that deletion of *upcY*, and its replacement with *ermF*, abolishes expression of the PSC gene cluster and illustrate the effectiveness of the allelic replacement methodology.

### Deletion of BF1708 encoding a putative polysaccharide copolymerase Wzz homologue

The presence or absence of polysaccharides in *B. fragilis* that resemble the classical O-antigen-containing LPS from *E. coli* has been the subject of much speculation (Poxton & Brown, 1986; Lindberg *et al.*, 1990; Lutton *et al.*, 1991). Dispersed in the NCTC 9343 genome are a number of 'orphan' genes that encode putative polypeptides with homology to proteins known to function in EPS biosynthesis. The original annotation of the genome identified two potential members of the polysaccharide copolymerase (PCP) family, encoded by BF1708 and BF2784 (Cerdeño-Tárraga *et al.*, 2005). No other proteins encoded by *B. fragilis* NCTC 9343 can be identified as PCPs using BLAST searches (E-value cutoff  $3 \times 10^{-4}$ ). The assignment of these two proteins as PCPs is supported by their current inclusion as the only proteins in *B. fragilis* within the InterPro003856 family, which contains 1897 members. PCPs are divided into three classes based on function and



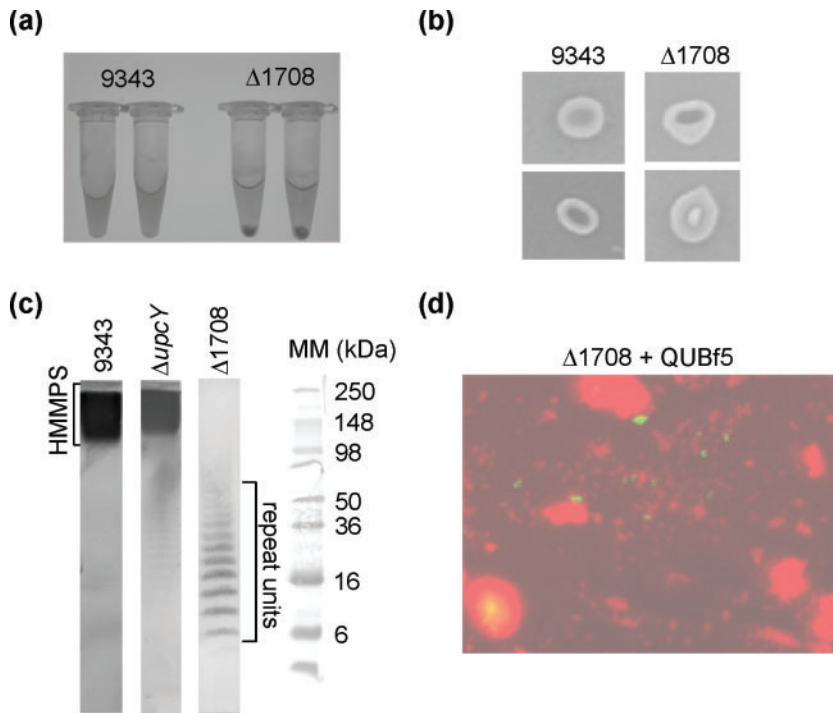


**Fig. 3.** Analysis of polysaccharide production in the  $\Delta upcY$  strain. (a) Examples of immunoblots probed with mAbs specific for polysaccharides produced by *B. fragilis* NCTC 9343 are shown on the left side, with the position of HMMPS indicated adjacent to the blots. Immunoblots of polysaccharides from the  $\Delta upcY$  strain are shown on the right side. A protein molecular mass ladder is provided to give an indication of the relative migration of polysaccharides during electrophoresis. mAbs which react with specific polysaccharides are: QUBf25 (PSB); QUBf5 (PSD); CE3 (PSA); QUBf7 (PSC). (b) Examples of IFM analysis of polysaccharides produced by the  $\Delta upcY$  strain. Panels on the left represent fluorescent labelling of mAbs QUBf18 (PSB), QUBf7 (PSC) and QUBf5 (PSD), while panels on the right show total cells in the same field of view stained with Evans blue. An example of IFM using QUBf7 labelling wild-type NCTC 9343 is shown at the bottom.

common secondary structural features (Morona *et al.*, 2000). Gene BF2784 encodes a polypeptide with homology to proteins of the PCP2 class, for example Wzc proteins that are involved in capsular biosynthesis (Whitfield, 2006). Gene BF1708, within PS gene cluster J/10, encodes a putative member of the PCP1 class, which includes Wzz proteins involved in O-antigen synthesis. Members of this family often show limited homology but have predicted N- and C-terminal transmembrane helices separated by a periplasmic coiled-coil region (Tocij *et al.*, 2008). The polypeptide encoded by BF1708 has 20 % identity to *E. coli* WzzB and is predicted to contain the secondary structure features associated with PCP1 members. In *E. coli* the Wzz protein modulates the polymerase activity of the Wzy involved in LPS biosynthesis to produce a distinctive modal distribution of O-antigen chain lengths (Franco *et al.*, 1998). Although BF1708 encodes the only identifiable homologue of Wzz in the NCTC 9343 genome, the high level of sequence divergence within the PCP classes means that other members might also be present.

To address the nature of the polysaccharide associated with LPS in *B. fragilis* and determine if the protein encoded by BF1708 was involved in LPS biosynthesis, we deleted the gene by replacing it with an *ermF* cassette and then analysed the polysaccharides produced by the mutant. The introduction of the *ermF* cassette could have a polar effect on expression of two downstream genes, BF1709 and BF1710; however, the products of these two genes are not likely to be central to polysaccharide production and are annotated as a putative transcriptional regulator and a protein of unknown function but with similarity to a dTDP-4-dehydrorhamnose 3,5-epimerase.

The first obvious phenotype of the  $\Delta 1708$  strain was the bridging flocculation of cells during culture in liquid medium (Fig. 4a), which may be indicative of altered cell-surface properties. Microscopy with negative staining for capsules revealed bacteria with either LC or SC, either individually or associated with the aggregates composed of bacteria non-capsulated by light microscopy. Labelling with a mAb specific for the SC confirmed its presence both by fluorescence microscopy and immunoblotting of polysaccharides (not illustrated). Examination of the capsule smears revealed an irregular capsular phenotype in approximately 10 % of the LC cells (Fig. 4b). This phenotype has not been observed previously in *B. fragilis* and how it relates to the deletion of BF1708 is open to speculation. Immunoblotting of MC polysaccharides produced by the  $\Delta 1708$  strain indicated that this culture was not antigenically mixed but was predominantly expressing a single polysaccharide that reacted with mAb QUBf5, which is specific for polysaccharides synthesized by the PSD locus (Fig. 4c). The blot pattern for the polysaccharide was, however, clearly distinct from that of the parental strain and other mutants, such as the  $\Delta upcY$  strain, which show HMMPS and an associated ladder similar to that previously described (Lutton *et al.*, 1991; Fig. 4c); in the  $\Delta 1708$  mutant the HMMPS was absent and



**Fig. 4.** Phenotypic analysis of the  $\Delta 1708/wzz$  strain. (a) Liquid cultures of *B. fragilis* NCTC 9343 and the  $\Delta 1708/wzz$  strain grown overnight. Note the aggregation of cells at the bottom of the tubes containing the  $\Delta 1708/wzz$  strain. (b) Negative staining of LC-producing cells from NCTC 9343 (left panels) and the  $\Delta 1708/wzz$  strain (right panels). Note the atypical protrusion of capsules from the  $\Delta 1708/wzz$  strain. (c) Immunoblots using QUBf5 (PSD) against polysaccharides derived from NCTC 9343,  $\Delta upcY$  and  $\Delta 1708/wzz$ . The relative positions of HMMPS and the smaller polysaccharide repeat units are shown adjacent to the blots. A protein molecular mass ladder is shown as an indication of the relative migration of polysaccharides during electrophoresis. (d) Combined IFM image of mAb QUBf5 labelling (green) and Evans blue total cell labelling (red) of the  $\Delta 1708/wzz$  strain. Note the presence of large aggregates of cells but the low-level detection of polysaccharides produced by the PSD locus.

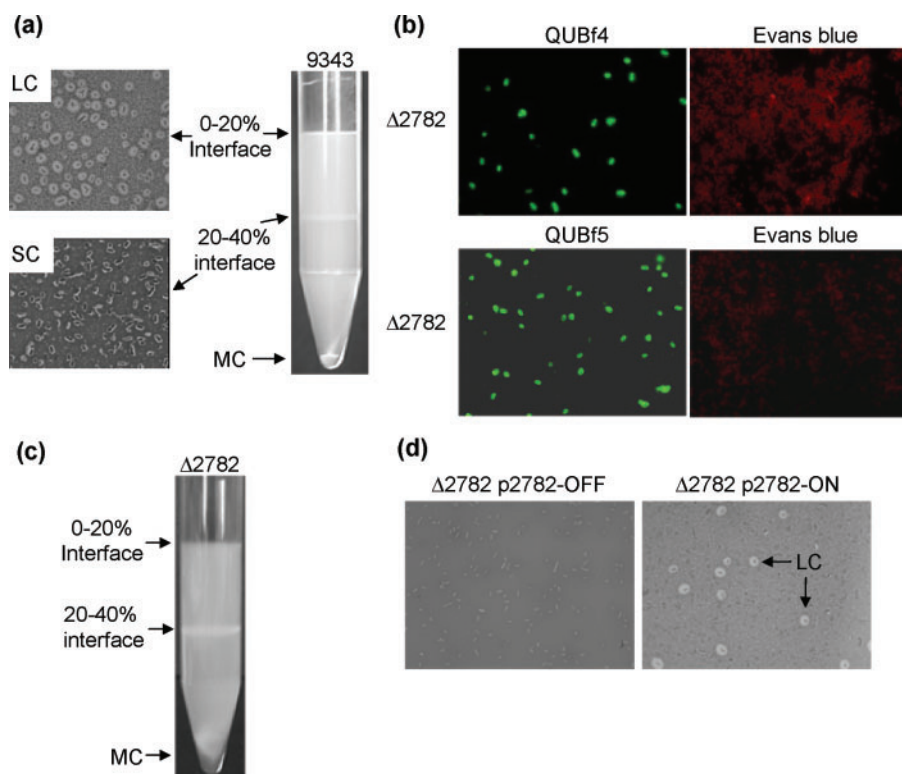
a lower-molecular-mass ladder pattern was evident (Fig. 4c). The loss of HMMPS D in the  $\Delta 1708$  strain is supported by the reduction in reactivity with mAb QUBf5 by IFM (Fig. 4d). The inability to detect HMMPS produced by the other loci, using immunoblotting and IFM, does not rule out the possibility that the polysaccharides are still present as short-chain repeats, which would reflect a reduced sensitivity of the mAbs for the residual repeat units. Together, these data indicate that deletion of the putative *wzz* gene and replacement with *ermF* prevents assembly of HMMPS synthesized by the PSD gene cluster into a long-chain-length polymer attached to the cell surface and that other HMMPS associated with the MC are not detectable.

#### Deletion of BF2782 encoding a putative polysaccharide export protein involved in synthesis of the large capsule

Phase variation with respect to capsule production in *B. fragilis* was first reported by Patrick & Reid (1983) when they described the separation of bacteria expressing capsules of different sizes using centrifugation through Percoll step gradients. While expression of seven EPS associated with the MC are regulated by site-specific recombination, little is known about the genes or the regulation of LC or SC production; clearly from the data presented above, the putative Wzz encoded by BF1708 is not involved. Annotation of the NCTC 9343 genome, however, identified a further set of orphan genes which are potentially involved in polysaccharide biosynthesis. ORFs BF2782 and BF2784 may encode homologues of the WbaP

and Wzc proteins, which are involved in group 1 and 4 capsule synthesis in *E. coli*. WbaP is part of a glycosyl-transferase complex involved in formation of lipid-linked precursors in the cytoplasm prior to translocation across the inner membrane, while Wzc (a member of the PCP2 family) is a membrane-bound protein that modulates the activity of the cognate Wzy polymerase. The promoter upstream of BF2782 was reported to be invertible and its orientation was reported to correlate with production of the LC, as shown by deletion of a tyrosine site-specific recombinase, *tsr19* (Chatzidaki-Livanis *et al.*, 2008). These authors, however, cultured their isolates in a complex basal medium that fails to reveal the LC phenotype and therefore does not distinguish between the antigenically different LC and SC. The LC and SC phenotypes are clearly evident on culture in defined medium (DM) by light microscopy (Fig. 1; Patrick & Reid, 1983; Patrick *et al.*, 1986) and are antigenically distinct (Reid *et al.*, 1987). LC and SC bacteria can be separated using a discontinuous Percoll density gradient where the LC population does not enter into the 20% Percoll, but remains at the top of the gradient after centrifugation, whereas the SC cells locate at the 20–40% interface layer (Fig. 5a). The MC cells pass through the gradient to the 60–80% interface (Patrick *et al.*, 1986). Capsule staining of wild-type cells from the 0–20% interface shows enrichment of cells expressing the LC, which are visually distinct from the SC population derived from the 20–40% interface (Fig. 5a). The Percoll gradients used to fractionate the tyrosine site-specific recombinase mutant illustrated by Chatzidaki-Livanis *et al.* (2008) show that the mutant with the 'locked ON' promoter has entered into the 20% Percoll suspension, and therefore it is not





**Fig. 5.** Phenotypic analysis of LC production in the  $\Delta 2782$  strain. (a) Discontinuous Percoll density gradient (20 and 40% Percoll) used to separate the LC, SC and MC populations of *B. fragilis* NCTC 9343 grown in defined medium. Micrographs showing negative staining of the antigenically distinct LC and SC capsules are presented on the left. The LC population remained on top of the 0–20% interface while the SC population remained at the 20–40% interface. (b) Examples of IFM images of mAb QUBf4 and QUBf5 labelling demonstrating that expression of SC and MC, respectively, is not altered in the  $\Delta 2782$  strain. (c) Discontinuous Percoll density gradient (20 and 40% Percoll) showing the fractionation of cells expressing the SC and MC from the  $\Delta 2782$  strain. There is no evidence of cells expressing the LC, which are expected to remain at the 0–20% interface. (d) Micrographs showing negative staining used to visualize LC production in the  $\Delta 2782$  strain containing plasmids with the promoter for BF2782 in either the 'OFF' or 'ON' orientation, grown in defined medium. Examples of cells expressing the LC in the complemented strain are indicated by arrows.

clear whether the LC or SC has been affected by the deletion. The negative staining of cells with the locked ON promoter reported by Chatzidaki-Livanis *et al.* (2008) also did not display a capsule that correlated with the previously described characteristics of the LC (Patrick & Reid, 1983; Patrick *et al.*, 1986; Reid *et al.*, 1987; Lutton *et al.*, 1991). To determine directly the role of BF2782 in production of either the LC or SC, we therefore generated both marked and markerless deletions of the gene. Replacement of BF2782 with an *ermF* cassette resulted in a mutant that was unable to synthesize the LC, as shown by the failure of cells to remain on top of 20% Percoll after centrifugation. This was confirmed by capsule staining of bacteria grown in DM followed by microscopy, which failed to detect any cells producing the LC (data not shown). IFM (Fig. 5b) and PAGE followed by immunoblotting (data not shown) of the  $\Delta$ BF2782 mutant using a suite of MC-specific mAbs indicated that deletion of BF2782 had not affected expression of the multiple-variable MC. Since insertion

of *ermF* may have been polar, we analysed the strain containing a markerless deletion of BF2782 using the same methods. The markerless mutant did not produce cells that remained on top of the 20% Percoll after centrifugation (Fig. 5c), and again we could not detect any cells expressing the LC by capsule staining (data not shown). SC bacteria were, however, evident at the 20–40% interface (Fig. 5c), and reaction with a mAb specific for the SC (Reid *et al.*, 1987) was positive by IFM (Fig. 5b). To confirm that the markerless deletion was responsible for the loss of LC production, we complemented the mutation by expressing BF2782 from a plasmid-borne copy of the gene. Two plasmids containing the BF2782 ORF were constructed: one had the invertible promoter locked ON while the other was always OFF. Only transformation of the  $\Delta 2782$  strain with the ON promoter construct enabled the cells to produce the LC, as determined by their presence on the 0–20% Percoll interface (data not shown) and capsule staining (Fig. 5d). Expression of the LC in this strain had

also returned to being phase variable, as only a fraction of the population produced the LC. This observation indicates that the invertible promoter on the chromosome of the markerless  $\Delta 2782$  strain was switching the expression of the remaining downstream gene(s) on and off, and implies that either BF2783 or BF2784, or both, are also involved in LC production.

## DISCUSSION

The nature of LPS in *B. fragilis* has been the subject of much speculation and controversy (reviewed by Patrick, 2002). It is clearly different from the paradigm of classical enteric LPS; for example, lipid A diglucosamine in *B. fragilis* is monophosphorylated rather than bisphosphorylated. While all the genes necessary to synthesize keto-deoxyoctonate (KDO) are present in the genome of NCTC 9343, its chemical composition is different from enteric KDO since it appears to be phosphorylated, thus rendering it undetectable in the standard thiobarbituric acid assay (Beckmann *et al.*, 1989). A major question, however, remains over the presence or absence of a polysaccharide that resembles an enteric O-antigen. Lindberg *et al.* (1990) stated that the repeating unit of an O-antigen is not present in *B. fragilis*, while Poxton & Brown (1986) clearly demonstrated that a polysaccharide with a repeat size equivalent to enteric O-antigen could be detected in at least one *B. fragilis* strain. Subsequently Lutton *et al.* (1991) described a within-strain antigenically variable HMMPS with an associated O-antigen-like ladder pattern after PAGE and immunoblotting, which was later designated PSD (Patrick *et al.*, 2003).

The genome of *B. fragilis* NCTC 9343 appears to be devoid of an operon with genetic structure similar to the loci involved in synthesis of O-antigen or lower-molecular-mass lipid A-linked K-antigen (termed  $K_{LPS}$ ) in *E. coli*. However, a single gene encoding a protein with homology to an O-antigen copolymerase, Wzz, was identified in *B. fragilis*. The Wzz protein in *E. coli* determines the modal distribution of O-antigen/ $K_{LPS}$  chain length, and deletion of the *wzz* gene simply produces unregulated lengths of polysaccharide chains. In group 1 and 4 K-antigens, for example, serotype K40, which forms both lipid A-linked  $K_{LPS}$  and high-molecular-mass non-lipid A-linked capsular (K)-antigen, there is no loss of higher-molecular-mass K-antigen when *wzz* is deleted. Deletion of BF1708 produced an unexpected phenotype whereby the HMMPS representing the MC were not detectable by immunoblotting using mAbs. This phenotype is similar to that described by Coyne *et al.* (2008) following deletion of a gene encoding a UDP-GlcNAc 4,6-dehydratase (BF1706), where the strain failed to produce polysaccharides associated with the MC and also underwent aggregation. It is hypothesized that the dehydratase is responsible for synthesizing the unusual sugar residues found in *B. fragilis* polysaccharides. Although both BF1706 and BF1708 seem to be involved in HMMPS biosynthesis, it is not clear if they are part of an operon since BF1706 is separated from BF1707 by 120 bp.

The loss of HMMPS in the *B. fragilis*  $\Delta wzz/1708$  mutant is very different from the change in distribution of O-antigen chain lengths in an *E. coli* *wzz* mutant, which was shown to be random and contained both larger and smaller numbers of repeat units compared to the wild-type (Franco *et al.*, 1998). The demonstration that a Wzz homologue is involved in controlling polysaccharide chain length also suggests that PSD and the other MC-associated polysaccharides may be covalently linked to lipid A-core. In group 1 or 4 capsules, lipid A-linked  $K_{LPS}$  usually only represents a small proportion of total capsular polysaccharide, with most being anchored to an unknown moiety on the cell surface as capsular or K-antigen. This would therefore make the antigenically variable MC-associated polysaccharides the equivalent of enteric LPS, but with a higher-molecular-mass polysaccharide that forms the electron-dense layer visible by electron microscopy on the exterior of the cell. Some strains of *E. coli* do produce very long-chain-length O-antigen polysaccharides, for example *E. coli* O157 (Sheng *et al.*, 2008); however, these polymers are still shorter than the HMMPS present in *B. fragilis* MC. A high-molecular-mass LPS in *B. fragilis* would be consistent with the findings of Lindberg *et al.* (1990). We also hypothesize that the one *B. fragilis* strain (NCTC 9344), which Poxton & Brown (1986) found to express LPS with short repeat units, potentially contained a mutation in *wzz*. This would be consistent with the short polymer repeat units that we observed on the immunoblots of the  $\Delta wzz/1708$  strain. Whether or not the MC polysaccharide is anchored to the lipid A, and therefore equivalent to a high-molecular-mass LPS, could be tested by deleting the gene encoding the ligase that attaches the polysaccharide to lipid A-core; however, homologues of *waaL* have yet to be identified in the genome. Nevertheless, the MC polysaccharide is present in LPS prepared using, for example, aqueous phenol extraction methods (Delahooke *et al.*, 1995).

Three different capsule types were originally identified in *B. fragilis*: MC, LC and SC (Patrick *et al.*, 1986). If the MC is anchored to lipid A-core and equivalent to a high-molecular-mass  $K_{LPS}$ , then are the LC and SC equivalent to *E. coli* groups 1 and 4, and related colanic acid, capsules? Association of mAb binding with expression of specific polysaccharide gene clusters has narrowed the focus to potential candidate genes involved in LC and SC formation. The genome sequencing project annotated three genes (BF2782–2784) encoding homologues of proteins potentially involved in group 1 and 4 capsule synthesis (Cerdeño-Tárraga *et al.*, 2005). An invertible promoter has been identified upstream of these three genes, and deletion of the tyrosine recombinase that mediates inversion of the promoter has allowed expression to be locked either in the ON or OFF conformation (Chatzidaki-Livanis *et al.*, 2008). The OFF position correlated with loss of capsule visible by light microscopy. These authors, however, used a complex medium in which the LC is not distinguishable from the SC by light

microscopy or Percoll gradient centrifugation (Patrick & Reid, 1983). We tested directly the hypothesis that BF2782 was involved in LC formation by deleting the gene and then complementing the mutation with a plasmid-borne copy of the gene. Deletion of BF2782 prevented the synthesis of the LC as determined by Percoll gradient centrifugation and capsule staining. The SC, however, was still expressed and this was confirmed by mAb labelling. Complementation of the non-polar markerless deletion resulted in phase-variable expression of the LC, even when BF2782 was constitutively expressed. This observation suggests that inversion of the promoter controlling expression of BF2783 and BF2784 in the  $\Delta$ 2782 strain was responsible for switching OFF expression and therefore implicates these genes in expression of the LC. The predicted protein encoded by BF2784 has homology to Wzc, which modulates the polymerase activity of the Wzy protein, and BF2783 has homology to Wza, a member of the outer-membrane auxiliary (OMA) protein family, which forms multimeric channels involved in polysaccharide extrusion. From these data we infer that assembly of the LC in *B. fragilis* is equivalent enzymically to production of colanic acid, group 1 and 4 K-antigen capsules in *E. coli*, whereas the MC are assembled by an entirely independent mechanism similar to that of K<sub>LPS</sub>. *B. fragilis* is therefore unusual not just in the number and variety of different polysaccharide capsule loci present in an individual strain, but also potentially in the nature of the LPS anchored at the cell surface. While the role of the LC in avoidance of phagocytic uptake and killing and the MC in resistance to serum killing has been reported (Reid & Patrick, 1984), the relative importance of these structures and the SC in survival in the human gastrointestinal tract remains to be determined.

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## REFERENCES

- Baughn, A. D. & Malamy, M. H. (2002). A mitochondrial-like aconitase in the bacterium *Bacteroides fragilis*: implications for the evolution of the mitochondrial Krebs cycle. *Proc Natl Acad Sci U S A* **99**, 4662–4667.
- Beckmann, I., van Eijk, H. G., Meisel-Mikolajczyk, F. & Wallenburg, H. C. (1989). Detection of 2-keto-3-deoxyoctonate in endotoxins isolated from six reference strains of the *Bacteroides fragilis* group. *Int J Biochem* **21**, 661–666.
- Begg, K. J. & Donachie, W. D. (1978). Changes in cell size and shape in thymine-requiring *Escherichia coli* associated with growth in low concentrations of thymine. *J Bacteriol* **133**, 452–458.
- Cerdeño-Tárraga, A. M., Patrick, S., Crossman, L. C., Blakely, G., Abratt, V., Lennard, N., Poxton, I., Duerden, B., Harris, B. & other authors (2005). Extensive DNA inversions in the *Bacteroides fragilis* genome control variable gene expression. *Science* **307**, 1463–1465.
- Chatzidaki-Livanis, M., Coyne, M. J., Roche-Hakansson, H. & Comstock, L. E. (2008). Expression of a uniquely regulated extracellular polysaccharide confers a large-capsule phenotype to *Bacteroides fragilis*. *J Bacteriol* **190**, 1020–1026.
- Coyne, M. J., Kalka-Moll, W., Tzianabos, A. O., Kasper, D. L. & Comstock, L. E. (2000). *Bacteroides fragilis* NCTC9343 produces at least three distinct capsular polysaccharides: cloning, characterization, and reassignment of polysaccharide B and C biosynthesis loci. *Infect Immun* **68**, 6176–6181.
- Coyne, M. J., Weinacht, K. G., Krinos, C. M. & Comstock, L. E. (2003). Mpi recombinase globally modulates the surface architecture of a human commensal bacterium. *Proc Natl Acad Sci U S A* **100**, 10446–10451.
- Coyne, M. J., Chatzidaki-Livanis, M., Paoletti, L. C. & Comstock, L. E. (2008). Role of glycan synthesis in colonization of the mammalian gut by the bacterial symbiont *Bacteroides fragilis*. *Proc Natl Acad Sci U S A* **105**, 13099–13104.
- Cruickshank, R. (1965). *Medical Microbiology: a Guide to the Laboratory Diagnosis and Control of Infection*, 11th edn, pp. 657–660. Edinburgh: E. & S. Livingstone Ltd.
- Delahooke, D. M., Barclay, G. R. & Poxton, I. R. (1995). A re-appraisal of the biological activity of *Bacteroides* LPS. *J Med Microbiol* **42**, 102–112.
- Dryden, D. T. (2006). DNA mimicry by proteins and the control of enzymatic activity on DNA. *Trends Biotechnol* **24**, 378–382.
- Fletcher, H. M., Schenkein, H. A., Morgan, R. M., Bailey, K. A., Berry, C. R. & Macrina, F. L. (1995). Virulence of a *Porphyromonas gingivalis* W83 mutant defective in the *prtH* gene. *Infect Immun* **63**, 1521–1528.
- Franco, A. V., Liu, D. & Reeves, P. R. (1998). The *wzz* (*clt*) protein in *Escherichia coli*: amino acid sequence variation determines O-antigen chain length specificity. *J Bacteriol* **180**, 2670–2675.
- Hoffman, L. M., Haskins, D. J. & Jendrisak, J. (2002). Type I inhibitor improves transformation efficiencies by blocking type I restriction and modification systems *in vivo*. *EPICENTRE Forum* **9**, 8.
- Krinos, C. M., Coyne, M. J., Weinacht, K. G., Tzianabos, A. O., Kasper, D. L. & Comstock, L. E. (2001). Extensive surface diversity of a commensal microorganism by multiple DNA inversions. *Nature* **414**, 555–558.
- Krüger, D. H., Schroeder, C., Hansen, S. & Rosenthal, H. A. (1977). Active protection by bacteriophages T3 and T7 against *E. coli* B- and K-specific restriction of their DNA. *Mol Gen Genet* **153**, 99–106.
- Krüger, D. H., Hansen, S. & Reuter, M. (1983). The *ocr*<sup>+</sup> gene function of bacteriophages T3 and T7 counteracts the *Salmonella typhimurium* DNA restriction systems SA and SB. *J Virol* **45**, 1147–1149.
- Li, L. Y., Shoemaker, N. B. & Salyers, A. A. (1995). Location and characteristics of the transfer region of a *Bacteroides* conjugative transposon and regulation of transfer genes. *J Bacteriol* **177**, 4992–4999.
- Lindberg, A. A., Weintraub, A., Zähringer, U. & Rietschel, E. T. (1990). Structure-activity relationships in lipopolysaccharides of *Bacteroides fragilis*. *Rev Infect Dis* **12** (Suppl. 2), S133–S141.
- Liu, C. H., Lee, S. M., Vanlare, J. M., Kasper, D. L. & Mazmanian, S. K. (2008). Regulation of surface architecture by symbiotic bacteria mediates host colonization. *Proc Natl Acad Sci U S A* **105**, 3951–3956.
- Lutton, D. A., Patrick, S., Crockard, A. D., Stewart, L. D., Larkin, M. J., Dermott, E. & McNeill, T. A. (1991). Flow cytometric analysis of

- within-strain variation in polysaccharide expression by *Bacteroides fragilis* by use of murine monoclonal antibodies. *J Med Microbiol* **35**, 229–237.
- Morona, R., Van Den Bosch, L. & Daniels, C. (2000). Evaluation of Wzz/MPA1/MPA2 proteins based on the presence of coiled-coil regions. *Microbiology* **146**, 1–4.
- Pantosti, A., Colangeli, R., Tzianabos, A. O. & Kasper, D. L. (1995). Monoclonal antibodies to detect capsular diversity among *Bacteroides fragilis* isolates. *J Clin Microbiol* **33**, 2647–2652.
- Patrick, S. (1993). The virulence of *Bacteroides fragilis*. *Rev Med Microbiol* **4**, 40–49.
- Patrick, S. (1997). Variation in *Bacteroides fragilis* surface structures. In *Anaerobic Pathogens*, pp. 19–30. Edited by A. R. Eley & K. W. Bennet. Sheffield: Sheffield Academic Press.
- Patrick, S. (2002). *Bacteroides*. In *Molecular Medical Microbiology*, pp. 1921–1948. Edited by M. Sussman. London: Academic Press.
- Patrick, S. & Duerden, B. I. (2006). Gram-negative non-spore forming obligate anaerobes. In *Principles and Practice of Clinical Bacteriology*, pp. 541–556. Edited by S. H. Gillespie & P. Hawkey. London: Wiley.
- Patrick, S. & Reid, J. H. (1983). Separation of capsulate and non-capsulate *Bacteroides fragilis* on a discontinuous density gradient. *J Med Microbiol* **16**, 239–241.
- Patrick, S. J., Reid, J. H. & Coffey, A. (1986). Capsulation of in vitro and in vivo grown *Bacteroides* species. *J Gen Microbiol* **132**, 1099–1109.
- Patrick, S., Stewart, L. D., Damani, N., Wilson, K. G., Lutton, D. A., Larkin, M. J., Poxton, I. & Brown, R. (1995). Immunological detection of *Bacteroides fragilis* in clinical samples. *J Med Microbiol* **43**, 99–109.
- Patrick, S., Parkhill, J., McCoy, L., Lennard, N., Larkin, M., Sczaniecka, M. & Blakely, G. (2003). Multiple inverted DNA repeats of *Bacteroides fragilis* that control polysaccharide antigenic variation are similar to the *hin* region inverted repeats of *Salmonella typhimurium*. *Microbiology* **149**, 915–924.
- Pósfai, G., Kolisnychenko, V., Bereczki, Z. & Blattner, F. R. (1999). Markerless gene replacement in *Escherichia coli* stimulated by a double-strand break in the chromosome. *Nucleic Acids Res* **27**, 4409–4415.
- Poxton, I. R. & Brown, R. (1986). Immunochemistry of the surface carbohydrate antigens of *Bacteroides fragilis* and definition of a common antigen. *J Gen Microbiol* **132**, 2475–2481.
- Redondo, M. C., Arbo, M. D., Grindlinger, J. & Snyderman, D. R. (1995). Attributable mortality of bacteremia associated with the *Bacteroides fragilis* group. *Clin Infect Dis* **20**, 1492–1496.
- Reid, J. H. & Patrick, S. (1984). Phagocytic and serum killing of capsulate and non-capsulate *Bacteroides fragilis*. *J Med Microbiol* **17**, 247–257.
- Reid, J. H., Patrick, S. & Tabaqchali, S. (1987). Immunochemical characterization of a polysaccharide antigen of *Bacteroides fragilis* with an IgM monoclonal antibody. *J Gen Microbiol* **133**, 171–179.
- Salysers, A. A., Bonheyo, G. & Shoemaker, N. B. (2000). Starting a new genetic system: lessons from *Bacteroides*. *Methods* **20**, 35–46.
- Sheng, H., Lim, J. Y., Watkins, M. K., Minnich, S. A. & Hovde, C. J. (2008). Characterization of an *Escherichia coli* O157:H7 O-antigen deletion mutant and effect of the deletion on bacterial persistence in the mouse intestine and colonization at the bovine terminal rectal mucosa. *Appl Environ Microbiol* **74**, 5015–5022.
- Simon, R., Priefer, U. & Pühler, A. (1983). Broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Biotechnology* **1**, 784–791.
- Sutherland, I. W. (1977). *Surface Carbohydrates of the Prokaryotic Cell*. London: Academic Press.
- Titheradge, A. J., King, J., Ryu, J. & Murray, N. E. (2001). Families of restriction enzymes: an analysis prompted by molecular and genetic data for type ID restriction and modification systems. *Nucleic Acids Res* **29**, 4195–4205.
- Tocilj, A., Munger, C., Proteau, A., Morona, R., Purins, L., Ajamian, E., Wagner, J., Papadopoulos, M., Van Den Bosch, L. & other authors (2008). Bacterial polysaccharide co-polymerases share a common framework for control of polymer length. *Nat Struct Mol Biol* **15**, 130–138.
- Valentine, P. J., Shoemaker, N. B. & Salysers, A. A. (1988). Mobilization of *Bacteroides* plasmids by *Bacteroides* conjugal elements. *J Bacteriol* **170**, 1319–1324.
- van Tassell, R. L. & Wilkins, T. D. (1978). Isolation of auxotrophs of *Bacteroides fragilis*. *Can J Microbiol* **24**, 1619–1621.
- Vimr, E. R. & Steenbergen, S. M. (2006). Mobile contingency locus controlling *Escherichia coli* K1 polysialic acid capsule acetylation. *Mol Microbiol* **60**, 828–837.
- Walkinshaw, M. D., Taylor, P., Sturrock, S. S., Atanasiu, C., Berge, T., Henderson, R. M., Edwardson, J. M. & Dryden, D. T. (2002). Structure of Ocr from bacteriophage T7, a protein that mimics B-form DNA. *Mol Cell* **9**, 187–194.
- Whitfield, C. (2006). Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Annu Rev Biochem* **75**, 39–68.

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